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β-Ionone-induced apoptosis in human osteosarcoma (U2os) cells occurs via a p53-dependent signaling pathway

Jiang Zhu · Lei Zhang · Xiaoming Jin · Xinying Han · Chuanhui Sun · Jinglong Yan

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Abstract β-Ionone is a constituent of vegetables and fruits, and can induce apoptosis in some types of malignant cells. However, the mechanism of apoptosis in osteosarcoma (U2os) cells is currently unclear. In this study, we determined whether β-ionone can induce apoptosis in U2os cells in vitro and which signal pathway(s) is involved. We found that β-ionone inhibited cell proliferation in U2os cells in a concentration- and time-dependent manner and caused cell cycle arrest at the G1-S phase. TUNEL assay, DNA ladder and assessment of Caspase 3 activity showed that apoptosis was the determinant in the effects of β-ionone. Furthermore, Expression of the p53 protein increased in a concentration-dependent and time-dependent manner according to immunocytochemistry and immunoblotting after β-ionone treatment. In addition, β-ionone upregulated Bax protein and downregulated Bcl2 protein which led to Bax translocation and cytochrome *c* release, subsequently activated Caspase 3, thus resulting in apoptosis. In summary, these data suggested that β-ionone induced apoptosis in a concentration-dependent manner in U2os cells via a p53-dependent mitochondrial pathway.

Keywords β-Ionone · U2os · Apoptosis · p53-Dependent mitochondrial pathway

Introduction

Osteosarcoma is a bone tumor that occurs predominantly in adolescents and young adults [1], and is the most common type of malignant bone cancer. The current treatment is to use neoadjuvant chemotherapy followed by surgical resection. The percentage of necrotic tumor cells are evaluated after surgery, and the prognosis and appropriate chemotherapy regimen are then assessed. Methotrexate, leucovorin, muramyl tripeptide, cisplatin, adriamycin, dactinomycin and etoposide are the most commonly used chemotherapeutic drugs for osteosarcomas.

Apoptosis is the goal of treating malignancies, and current studies have focused on finding novel and effective drugs that can induce and enhance apoptosis, and which is an ideal strategy for the treatment of osteosarcomas.

Epidemiological studies have demonstrated that high intake of plant foods, including fruits and vegetables, can prevent the occurrence of tumors and decrease the risk of cancer. Plant foods contain high levels of nutrients and physiologically active phytochemicals [2]. Nutritional supplements such as raspberries, tomatoes and roses, which are rich in β-ionone compounds, have been used because of their anticancer properties [3].

β-Ionone is known to exert chemopreventive effects on cancers by inducing cell apoptosis; for example, β-ionone has been shown to induce apoptosis in gastric adenocarcinoma cells and endothelial cells [4, 5]. β-Ionone is also a precursor for carotenoids, which exert anticancer and antineoplastic effects in melanoma, mammary adenocarcinoma, meningioma cells and colon carcinoma [6–10].

Drs. Jiang Zhu and Lei Zhang are contributed equally to this work.

J. Zhu · J. Yan (✉)
Department of Orthopedics, The First Affiliated Hospital of Harbin Medical University, Harbin Medical University, # 23, Youzheng Street, 150001 Nangang District, Harbin, Heilongjiang Province, People's Republic of China
e-mail: yanjinglong2009@yahoo.cn

L. Zhang · X. Jin · X. Han · C. Sun
Department of Pathology, Harbin Medical University, Harbin, Heilongjiang Province, People's Republic of China

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The molecular mechanisms of β-ionone-induced apoptosis in osteosarcoma cells remain unclear. This study was undertaken to investigate how β-ionone induces apoptosis in human osteosarcoma cells and to deduce the molecular mechanisms involved. To evaluate the potential function of β-ionone, cultured p53-positive cell line (U2os) and p53-negative cell line (Saos2) were used in the present study to demonstrate the apoptotic function and to determine the mechanisms involved in osteosarcoma cells.

We used growth curve analysis, cell cycle analysis, morphological observation under light microscope, transmission electron microscopy and TUNEL assay to examine the anticancer activity of β-ionone. We also used immunoblotting and immunocytochemistry to characterize the signaling pathway involved in β-ionone-induced apoptosis in human osteosarcoma U2os cells. Of interest, β-ionone was unable to induce apoptosis in osteosarcoma Saos2 cells.

Materials and methods

Cell culture

U2os and Saos2 cells derived from human osteosarcoma were obtained from the cell bank of the Chinese Academy of Sciences and grown in DMEM-F12 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco Laboratories), 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were incubated in a humidified 5% CO_2 incubator at 37°C.

Drug treatment

β-Ionone was dissolved in ethanol and used for the treatment of U2os and Saos2 cells. The final concentration of ethanol used was 0.1% (v/v) for each treatment. Control cells were treated with ethanol alone.

Determination of cell viability

The cytotoxicity of β-ionone was determined by the MTT assay. U2os and Saos2 cells were seeded on 96-well, flat-bottomed culture plates at 5×10^3 cells/well. The U2os and Saos2 cells were incubated for 48 h with various concentrations of β-ionone, ranging from 50 to 400 μM, and U2os cells were incubated with 175 μM β-ionone for 12, 24, 36, 48, 60, 72, or 96 h. Then, 20 μl MTT (5 mg/ml) was added to each well and incubated for 3 h at 37°C. The culture medium was removed and replaced with 150 μl of DMSO to dissolve the purple formazan product and the absorbance was measured on a spectrophotometer microplate reader (Bio-rad Model 550) at a wavelength of 570 nm.

Analysis of cell cycle arrest by flow cytometry

Flow cytometry and propidium iodide staining were used to determine the different phases of the cell cycle. After exposure to β-ionone for 48 h, the U2os cells were harvested, washed three times with phosphate-buffered saline (PBS), centrifuged (1,500 rpm for 10 min), and fixed in 75% (v/v) ethanol at 4°C for 24 h. Cells (10^6) were pelleted by centrifugation (1,500 rpm for 10 min), washed once with PBS, and resuspended in propidium iodide solution (20 mg/ml propidium iodide and 0.2 mg/ml RNase A in PBS [pH 7.4]) for 30 min at room temperature in the dark. Red fluorescence for each well was recorded using an argon ion laser with an excitation wavelength of 488 nm and an emission wavelength of 610 nm to measure the DNA index. Flow cytometric analysis was done on a FACS Canto Flow Cytometer (Becton Dickinson). Flow cytometry plots were prepared to display the number of cells on the ordinate and the DNA content on the abscissa. Data are representative of three experiments.

Observation of morphological changes

To confirm whether β-ionone induces apoptosis in U2os cells, we investigated the morphological change of U2os cells after treatment with β-ionone. Cells were treated with 50, 100 or 200 μM β-ionone for 48 h and were observed under a light microscope and photographed.

Preparation of samples for transmission electron microscopy

To further confirm whether β-ionone induces apoptosis in U2os cells, the cells were observed under a transmission electron microscope (TEM); samples were prepared as described below. U2os cells were incubated for 48 h with various concentration β-ionone, harvested and washed twice with PBS. Cells (10^6) were pelleted by centrifugation (1,500 rpm for 10 min) and fixed in 3% (v/v) glutaraldehyde for 24 h at 4°C. Cells were washed three times with 0.18 M sucrose in 0.1 M phosphate buffer. Cells were then fixed in 1% OsO_4 for 2 h at 4°C. After dehydration in a graded series of ethanol (15 min each) and embedding in Epon 812 at 37°C overnight, the cells were cut into sections using a Leica Ultracut R cutter. After staining with uranyl acetate and lead citrate, the sections with apoptotic nuclei were observed and photographed under a JEM-1010 transmission electron microscope.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay

Adherent U2os cells were treated with various concentration β-ionone (50, 100 or 200 μM) for 48 h and or with

200 μM β-ionone for 12, 24, 36 or 48 h were fixed in 4% paraformaldehyde in PBS for 10 min at room temperature, and permeabilized with 0.1% triton X-100 in 0.1% sodium citrate for 2 min on ice. The cells were then incubated with TUNEL reaction mixture for 1 h at 37°C. Five hundred cells in each section were counted under fluorescence microscopy (Nikon E800) to calculate the positive ratio of TUNEL staining. For each section, ten separate fields of view were captured randomly and assessed by a pathologist. Cells with clear nuclear labeling were defined as TUNEL-positive cells. Six sections from each concentration were stained and measured.

DNA ladder

To analyze DNA fragmentation, 5×10^6 U2os cells were treated with 200 μM β-ionone for 12, 24, 48 or 72 h and were incubated with 4 ml cytolytic buffer (25 mM EDTA, 100 mM NaCl, 10 mM Tris–HCl, 0.5% SDS, 0.2 mg/ml protease K, pH 8.0) for 4 h at 56°C. The cell lysate was then extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and centrifuged for 20 min. The supernatant was incubated at 37°C for 60 min with RNase (0.2 μg/ml) (Sigma). Then, it was extracted with phenol and chloroform (1:1) and centrifuged at 12,000 rpm for 5 min. Finally, it was centrifuged in the presence of the same volume of ice-cold ethanol (100%) at 12,000 rpm for 5 min. The pellets were washed with ice-cold ethanol (70%) and dried. The DNA in pellets was resolved by 100 μl TE buffer at 4°C overnight. Electrophoresis was performed at 60 V with 2% agarose gels containing ethidium bromide and was visualized under UV light.

Caspase-3 cellular activity assay

The cells were cultured on 75 cm^2 plates, and treated with various concentration β-ionone (50, 100 or 200 μM) for 48 h or with 200 μM β-ionone for 0, 6, 12, 24, 48 or 72 h. Cells were counted and harvested by centrifugation (1,000 rpm, 4°C, 10 min). The cells (2×10^7/ml) were then incubated for 5 min in ice bath, treated with ice-cold cell lysis buffer (50 mM HEPES, pH 7.4, 0.1% CHAPS, 5 mM DTT, 0.1 mM EDTA) and then centrifuged at 10,000 rpm for 10 min at 4°C. The supernatants were retained and placed on ice until used. The protein concentration was determined with the BCA Protein Assay (Beyotime Institute of Biotechnology) with bovine serum albumin (BSA) as a standard. Then, 30 mg of total protein was added to a 96-well microplate, and the reaction was started by the addition of 50 μl of Ac-DEVD-pNA. The amount of pNA was determined using a microplate reader at an absorbance of 405 nm, and data were recorded twice at intervals of 10–60 min. The caspase 3 activity was calculated according to the manufacturer's instructions (Caspase-3 Cellular Activity Assay Kit Plus, BIOMOL Research Laboratories Inc., PA, USA). The data are expressed as mean values ± standard deviation for three independent experiments.

Immunocytochemistry

Glass coverslips were placed in 24-well plates, and U2os cells were seeded in the 24-well plates filled with 1 ml of culture medium to a concentration of 1×10^4/ml, and incubated at 37°C in a 5% CO_2 incubator. After culture for 24 h, the U2os cells were treated with various concentration β-ionone (50, 100 or 200 μM) for 48 h. The plates were then washed briefly in PBS. Adherent cells were fixed in 95% ice-cold ethanol for 30 min at room temperature and washed twice with ice-cold PBS, incubated for 10 min with PBS containing 0.25% Triton X-100 to permeabilize the cells, washed in PBS, incubated with 10% goat serum for 30 min to block non-specific antibody binding, and incubated with the diluted primary antibody (p53, Cytochrome *c*, Bax or Bcl2) in 1% BSA in PBS in a humidified chamber at 4°C overnight, then incubated with the secondary antibody in 1% BSA for 30 min at room temperature and the chromogenic reaction was done with diaminobenzidine (DAB) staining. The proportion of cells expressing p53 was calculated as for the TUNEL assay. The results were analyzed with a light microscope. The monoclonal anti-human p53 (hp53), monoclonal anti-h Cytochrome *c*, and polyclonal anti-h Bax antibodies were purchased from Santa Cruz Biotechnology, CA, and the monoclonal anti-Bcl2 (Ab-1) antibody was purchased from Calbiochem Novabiochem Corporation, La Jolla, CA. Six sections from each concentration were stained and measured.

Protein extraction and Western blotting

Cells were cultured in 10 cm diameter dishes for 48 h at 37°C; then, the cells were treated with various concentration β-ionone (50, 100 or 200 μM) for 48 h. The cell culture dishes were placed on ice, and the cells were washed with ice-cold PBS three times. The PBS was removed and lysis buffer (RIPA buffer: 50 mM Tris–HCl,150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 μg/ml aprotinin, 5 μg/ml leupeptin, 2 μg/ml antipain, 1 mM PMSF) was added to each dish. Adherent cells were scrapped off the dish with a cold plastic cell scraper and the cell suspension was placed in a pre-cooled Eppendorf tube. The Eppendorf tubes were kept on ice for 1 h, and centrifuged in a refrigerated centrifuge at 12,000 rpm for 40 min. The protein concentration in the supernatant fraction was measured with the BCA Protein Assay (Beyotime Institute

of Biotechnology) using BSA as a standard. Protein samples (50 μg) in 1× loading buffer were boiled at 95–100°C for 10 min to denature the protein. The samples were subjected to 12 or 15% SDS–polyacrylamide gel electrophoresis. The separated proteins were transferred onto PVDF membranes in ice-cold transfer buffer (transfer buffer: 0.15 M Tris–HCl, 0.2 M glycine, 10% methanol). The membranes were blocked with 5% blocking serum overnight at 4°C and incubated with the primary antibody at the suggested dilution (p53: 1:1,000 dilution; polyclonal antibody anti-hpCaspase 3 (Santa Cruz Biotechnology, CA): 1:500 dilution; Bax: 1:1,000 dilution; Bcl2: 1:1,000 dilution) in Tris-buffered saline (TBS) with Tween-20 (TBST) at room temperature for 2 h, and the membranes were washed three times with TBST. The membranes were incubated with appropriate concentrations of alkaline phosphatase-conjugated secondary antibodies for 2 h at room temperature and the membranes were washed twice with TBS. BCIP/NBT detection kits (Sigma) were used to detect the antigen. The protein level was measured using ScnImage software.

Statistical analysis

In the study, all data shown are means ± standard error of the mean. Statistical differences between the treated groups and controls were calculated using one-way ANOVA. Significance was accepted at $P < 0.05$.

Results

Effects of β-ionone on cell viability

The IC50 value was calculated from the 50% formazan formation compared with a control without administration of β-ionone. The MTT assay was used to detect cell viability. The viability of U2os cells was remarkably decreased in a dose-dependent manner after treatment with doses of β-ionone of 50, 100, 150, 200, 250, 300, 350 and 400 μM for 48 h. The cytotoxicity of β-ionone according to the IC50 value was approximately 175 μM (Fig. 1a). For Saos2 cells, the survival rate at 400 μM was 92% (Fig. 1a). We also assessed the time-dependent effect of β-ionone on cell viability of U2os cells at the IC50 concentration of 175 μM, and the cell viability was less than 15% after β-ionone exposure for 60 h (Fig. 1b). All measurements and each experiment were repeated at least three times.

Determination of cell cycle arrest by flow cytometry

The effect of β-ionone on the cell cycle of U2os cells was detected by flow cytometry. β-ionone suppressed proliferating cells at the G1 phase. The cell counts in S phase were significantly decreased after treatment with 200 μM β-ionone for 48 h (Fig. 1d). Accordingly, β-ionone can inhibit U2os cell proliferation and induce cell cycle arrest.

Observation of apoptosis under a light microscope and TEM

We found that the number of cells decreased after β-ionone treatment and the shape of cells changed, exhibiting cell shrinkage and cytoplasmic membrane blebbing under light microscopy (Fig. 2a). The apoptotic cells showed marked shrinkage and deformation, nuclear chromatin condensation, chromosomal DNA fragmentation, nuclear fragmentation and increased numbers of apoptotic bodies. Cell shrinkage and fragmentation-formed apoptotic bodies with dense chromatin were also observed under TEM (Fig. 2b).

TUNEL assay

The proportion of apoptotic cells was calculated as the percentage of TUNEL positive cells using the following formula: number of TUNEL positive cell nuclei/number of total cell nuclei × 100%.

The apoptotic bodies were observed using the TUNEL assay. The proportion of apoptotic cells was significantly increased by β-ionone treatment (Fig. 3a). The number of TUNEL-positive cells increased in a concentration dependent manner, the proportion of positive cells increased from 11.52 ± 1.62% with 50 μM β-ionone, to 17.38 ± 1.75% with 100 μM β-ionone, and reached 43.43 ± 6.57% with 200 μM β-ionone (Fig. 3b) ($P < 0.01$, $n = 6$). The proportion of TUNEL-positive cells increased in a time-dependent manner after 200 μM β-ionone treatment (Fig. 3c) ($P < 0.01$, $n = 6$).

DNA ladder

DNA ladder formation in U2os cells was observed after treatment with 200 μM β-ionone for 12, 24, 48 or 72 h. Cells treated with 200 μM β-ionone for 48 h exhibited significant formation of oligonucleosomal fragments; thus, 48 h was considered an optimal time-point for assessing apoptosis (Fig. 3d).

β-Ionone promotes the expression of Caspase 3

To determine the mechanism of β-ionone in apoptosis of U2os cells, we assessed the activity of Caspase-3 as a critical point in the apoptotic pathway. Caspase-3 cellular activity increased in a dose- and time-dependent manner in response to β-ionone treatment. The Caspase-3 cellular activity showed a significant increase in cells treated with 100 and 200 μM β-ionone ($P < 0.01$, $n = 3$) compared

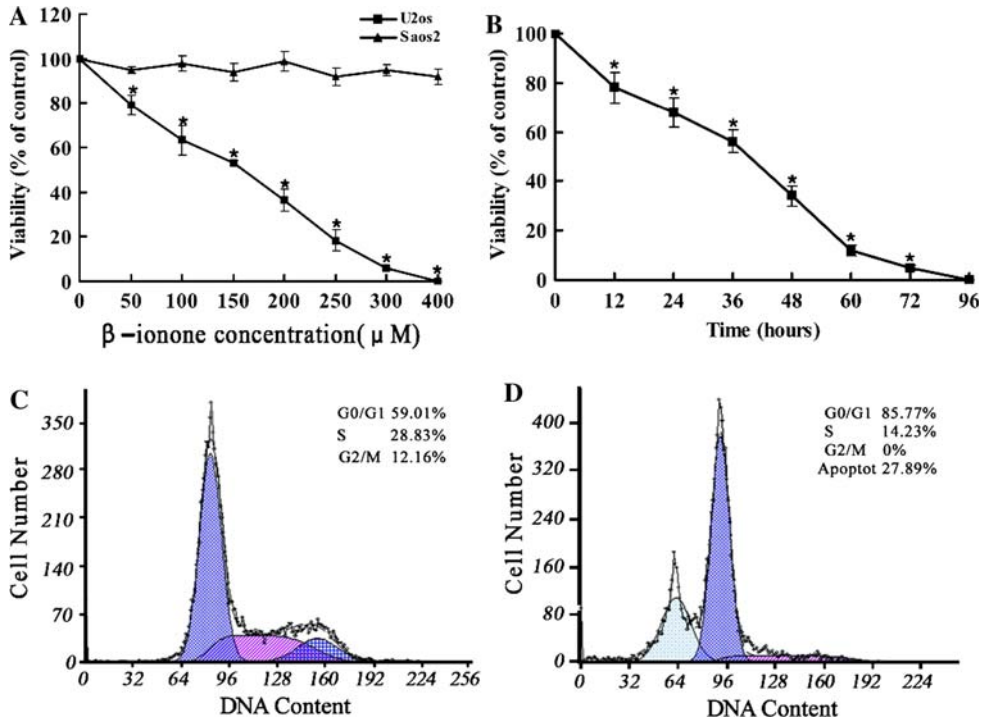

Fig. 1 **a** Cytotoxic effects of *β*-ionone on U2os cells and Saos2 cells. Cells were seeded for 12 h before the addition of *β*-ionone with various concentrations. Cells were treated with different concentration of *β*-ionone, ranging from 50 to 400 μM for 48 h. Cell viability was determined with MTT assay and compared with cells incubated with ethanol only. *Control* value was regarded as 100%. Data are expressed as mean values ± standard deviation of three independent experiments. **b** *β*-Ionone inhibited the proliferation of U2os cells. The figure shows the cell viability of U2os cells treated with the IC50 dose (175 μm) *β*-ionone for various times. The MTT assay was used to measure cell viability. Data are expressed as mean values ± standard deviation of three independent experiments. **c**, **d** Flow cytometry analysis. Propidium iodide staining of U2os cells treated with ethanol *control* (**c**) or 200 μM (**d**) *β*-ionone for 48 h. *β*-Ionone induced cell cycle arrest at the G1 stage. Flow cytometry plots display the number of cells on the ordinate and the DNA content on the abscissa. Data are representative of three experiments

with cells treated without or with 50 μM *β*-ionone (Fig. 4a). The Caspase-3 cellular activity showed a significant increase in cells treated with 200 μM *β*-ionone for 48 h compared with cells treated with *β*-ionone for 12 or 24 h (Fig. 4b). Because the activity of Caspase-3 was significantly increased in U2os cells treated with 200 μM *β*-ionone for 48 h, this treatment was considered to represent the optimal induction condition of apoptosis (Fig. 4c). U2os cells were treated with *β*-ionone at various concentrations of 50, 100 or 200 μM for 48 h and the expression of active Caspase 3 (17 kDa) was enhanced after *β*-ionone treatment at concentrations of 100 or 200 μM for 48 h (Fig. 4d).

β-Ionone promotes the expression of p53 and Bax but inhibits the expression of Bcl2

To determine the pathway by which *β*-ionone induces apoptosis in U2os cells, we performed immunoblotting for p53, Bax and Bcl2. Treating U2os cells with *β*-ionone for 48 h induced the expression of p53 and Bax in a dose-dependent manner, whereas the expression of Bcl2 was decreased in a dose-dependent manner (Fig. 5).

The immunocytochemical results of p53 showed that the expression of p53 was increased by *β*-ionone in dose-dependent manner over 48 h. The p53 protein was predominantly expressed in the nucleus (Fig. 6a). The proportion of p53-positive cells increased from 3.82 ± 1.25% with ethanol control, to 31.49 ± 4.62% with 50 μM *β*-ionone and 54.83 ± 4.78% with 100 μM *β*-ionone, reaching 86 ± 4.69% with 200 μM *β*-ionone. The results were in accordance with the immunoblotting for p53 (Fig. 6b). Besides, p53 protein was detected in U2os cells that incubated with 200 μM *β*-ionone for 12, 24, 36, and 48 h, the expression of p53 was increased by *β*-ionone in time-dependent manner (Fig. 6c).

Our immunocytochemistry findings for Bax and Bcl2 revealed that *β*-ionone increased the expression of Bax but decreased the expression of Bcl2 (Fig. 7a, b). The Bax protein was relatively concentrated in the mitochondria in U2os cells after 200 μM *β*-ionone treatment for 48 h; in contrast, Bax diffusely expressed in the cytosol in control cells (Fig. 7b).

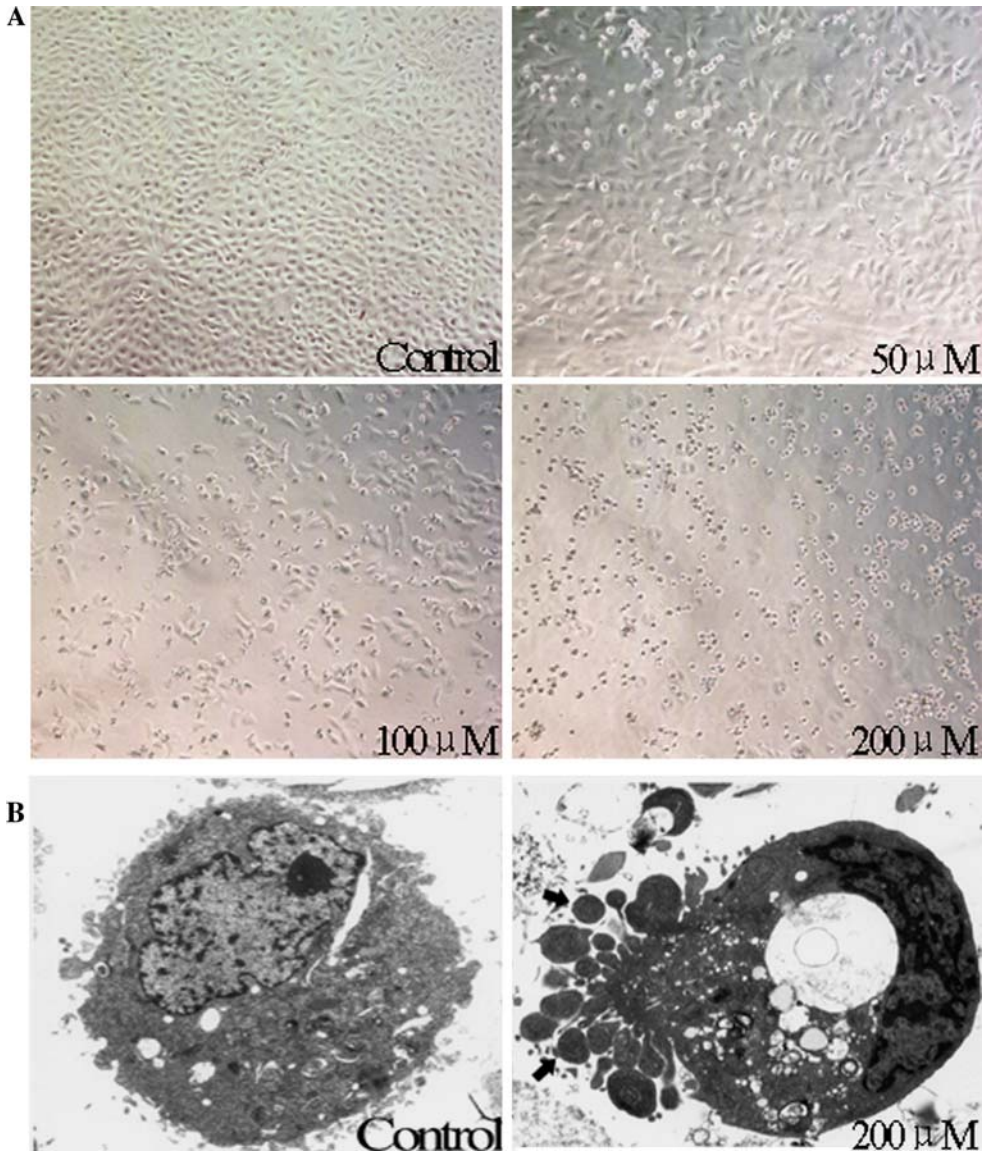

Fig. 2 **a** Morphological changes of U2os cells in response to β-ionone treatment. Cells were treated with 50 μM β-ionone, 100 or 200 μM β-ionone for 48 h and were observed under a light microscope. The number of cells decreased after β-ionone treatment and the cells exhibited cell shrinkage and cytoplasmic membrane blebbing (magnification: ×200). **b** Morphological change of U2os cells in response to β-ionone treatment. Cells were treated with 50, 100 or 200 μM β-ionone for 48 h and were observed under a transmission electron microscope. The *right* figure indicates marked shrinkage and deformation, nuclear chromatin condensation, chromosomal DNA fragmentation, nuclear fragmentation and increased apoptotic bodies in the apoptotic cells. Cell shrinkage and fragmentation-formed apoptotic bodies with dense chromatin are indicated. (*Arrows*: apoptotic bodies; magnification: ×5,000)

The release of cytochrome *c* from mitochondria

Cytochrome *c* is released from the mitochondria in response to β-ionone via Bcl2 family-regulated mechanisms. The release of cytochrome *c* is a characteristic of cellular apoptosis. In U2os cells treated with β-ionone for 48 h, the cytochrome *c* protein was relatively diffusely expressed in the cytosol; in contrast, cytochrome *c* was relatively concentrated in the mitochondria in control cells (Fig. 7c).

Discussion

Several epidemiologic studies have consistently shown that high intake of fruits and vegetables confers a protective effect against various types of cancer [11]. β-ionone is widely distributed in fruits and vegetables, and has been shown to induce apoptosis in some types of malignant tumors [8, 12, 13]. The apoptotic effect of β-ionone in osteosarcoma is not well defined; therefore, the objective of this study was to evaluate the apoptotic effects of β-ionone

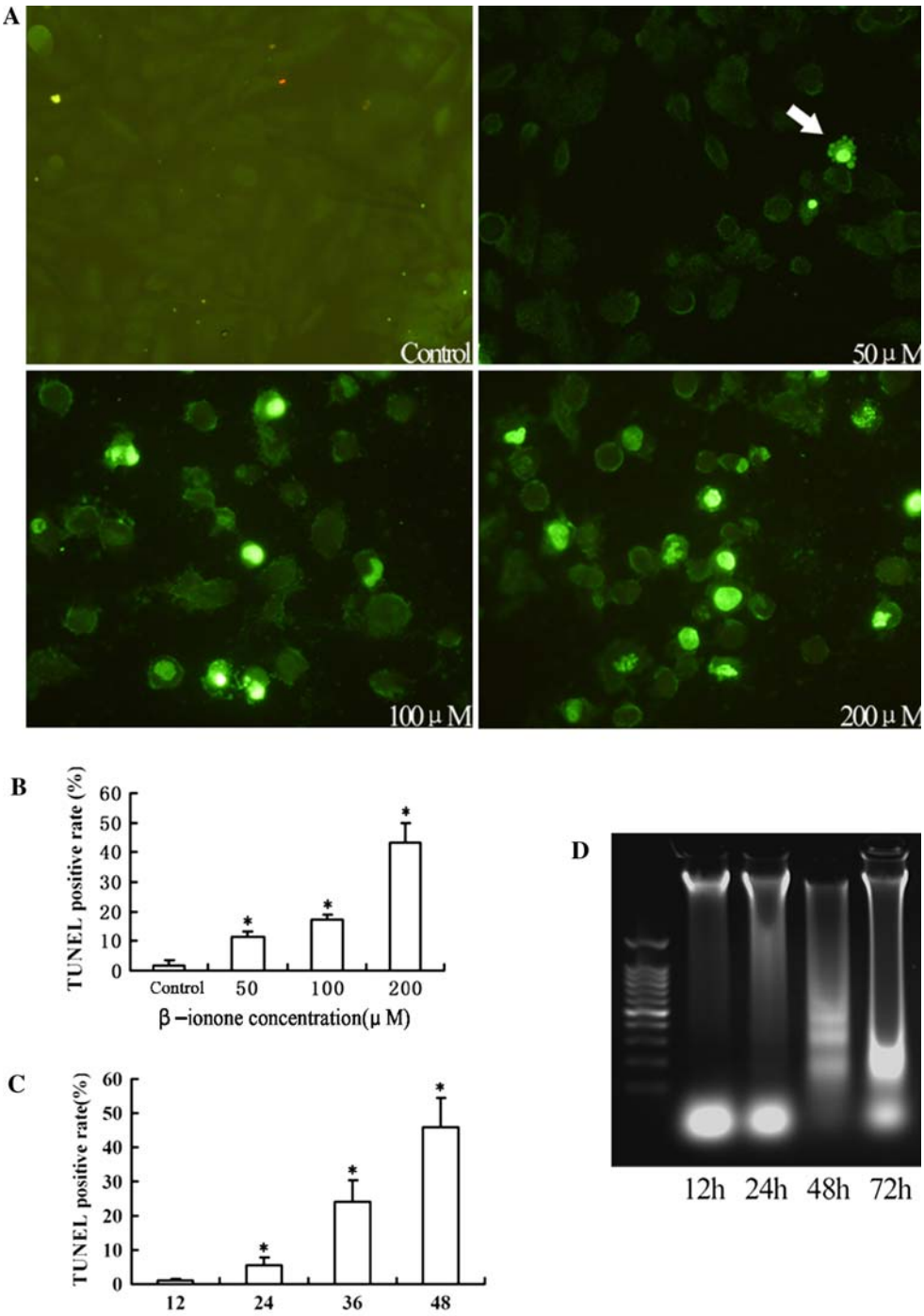

Fig. 3 **a**, **b**, **c** The numbers of apoptotic bodies were determined using TUNEL assay. The number of TUNEL-positive cells was significantly increased by β-ionone in a dose- and time-dependent manner (*arrows*: apoptotic cells; magnification: $\times 200$). Data are expressed as mean values $\pm$ standard deviation of six independent experiments ($P < 0.01$). **d** DNA ladder formation in U2os cells treated with 200 μM β-ionone for 12, 24, 48 or 72 h. The formation of oligonucleosomal fragments was significant at 48 h, which was considered to indicate the optimal time-point for assessing apoptosis

and to determine the apoptotic pathway in osteosarcoma cells.

In previous studies, β-ionone was found to inhibit the growth of gastric cancer, colon and breast cancers, inducing the apoptosis of SGC7901 [4], HCT116 [13], and MDA-MB cells [14], with IC50s of 89, 60 and 42.0 μM, respectively, after exposure to β-ionone for 24 h. However, in our study, the IC50 of β-ionone in U2os cells was 175 μM at 48 h, and the cytotoxic effects of β-ionone on U2os cells were lower than the above-mentioned tumor cells indicated that U2os cells showed stronger drug resistance. In addition, we chose 48 h for the treatment duration of β-ionone in U2os cells because the morphological changes, TUNEL assay, DNA ladder and Caspase 3 activity were most marked at this time. This treatment duration is longer than that in the above-mentioned cells.

It is well known that p53 is a multi-functional transcription factor that regulates critical cellular processes, including proliferation, cell cycle arrest, DNA repair and apoptosis. p53 can induce cell cycle arrest in the G1, G2 or

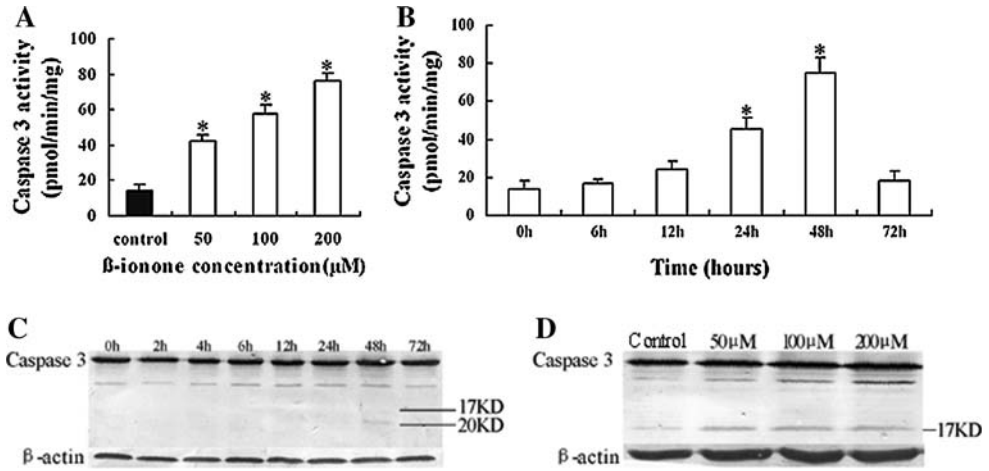

Fig. 4 Caspase 3 activation in U2os cells treated with β-ionone. **a** The Caspase 3 activity increased dose dependently at β-ionone concentrations of 50, 100 and 200 μM for 48 h ($n = 3$, $P < 0.01$). **b** Caspase 3 activity increased in a time-dependent manner from 0 to 48 h with 200 μM β-ionone and was significantly increased at 48 h ($n = 3$, $P < 0.01$). **c** Western blot analysis of Caspase 3 protein expression at different times after treatment with 200 μM β-ionone. The expression of the active form of Caspase 3 (17 and 20 kDa) was significantly increased at 48 h. **d** Western blot analysis of Caspase 3 protein expression after treatment with various concentrations of β-ionone (50, 100 and 200 μM) for 48 h. Expression of the active form of caspase 3 (17 kDa) increased significantly

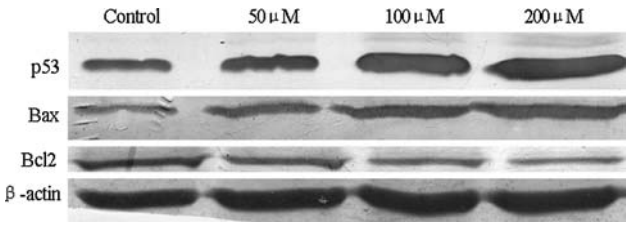

Fig. 5 Western blot analysis of the expression of p53, Bax and Bcl2 in U2os cells treated with 50, 100 or 200 μM β-ionone for 48 h. β-ionone increased the expression of p53 and Bax and decreased the expression of Bcl2

S phases to repair genomic damage [15]. We used a p53-positive cell line (U2os) and a p53-negative cell line (Saos2) as models to observe the effect of β-ionone on cell proliferation. We found that Saos2 cells were largely unaffected by β-ionone, and thus our study focused on U2os cells. The cytotoxicity assays revealed that β-ionone induced the death effect of U2os cells in a dose- and time-dependent manner.

In the next sets of experiments, the apoptotic function of β-ionone on U2os cells was detected by the morphological change observation, Tunnel assay and DNA ladder. Apoptosis was clearly evident in U2os cells treated with β-ionone for 48 h. Using TUNEL assay, we found that the number of apoptotic bodies increased in direct proportion with the concentration of β-ionone. The proportion of apoptotic bodies increased slightly at 100 μM β-ionone, and was particularly high at the concentration of 200 μM. The results of the DNA ladder assessment showed that U2os cells treated with 200 μM β-ionone for 48 h exhibited significant formation of oligonucleosomal fragments.

In our study, β-ionone promoted p53 protein expression in U2os cells. Expression of the p53 protein in U2os cells increased in a concentration-dependent and time-dependent manner according to immunocytochemistry and immunoblotting after β-ionone treatment. In addition, we observed that p53-negative cells (Saos2), compared with p53-positive cells (U2os cells), were resistant to β-ionone-induced apoptosis. These observations suggest p53 maybe the critical factor in the apoptotic process in U2os cells after β-ionone treatment and β-ionone induces p53-sensitive cells apoptosis.

In our study, U2os cell cycle arrest at G1-S stage under the β-ionone -induced and p53-mediated effects. If the DNA damage is extensive and the repair capability is not enough, the apoptotic pathway is triggered. Then, p53 can activate and inhibit some downstream genes to initiate apoptosis, including Bcl2 family members. Bcl2 family proteins are known to modulate apoptosis via mitochondrial apoptosis pathway [16]. Bcl2 and Bax are two members of the Bcl2 protein family, which regulate the balance between cell proliferation and apoptosis. The ratio of Bcl2 and Bax is proportional to the relative sensitivity or resistance to a wide variety of apoptotic stimuli [17]. Anti-apoptotic Bcl2 is mostly localized to the outer mitochondrial membrane, endoplasmic reticulum membrane and outer nuclear membrane, to block the release of cytochrome *c* from mitochondria. However, Bax is a pro-apoptotic protein, and induces the opening of mitochondrial voltage-dependent anion channels, which results in the release of cytochrome *c* and other pro-apoptotic factors from the mitochondria, leading to activation of downstream Caspases. To further confirm the molecular mechanisms of

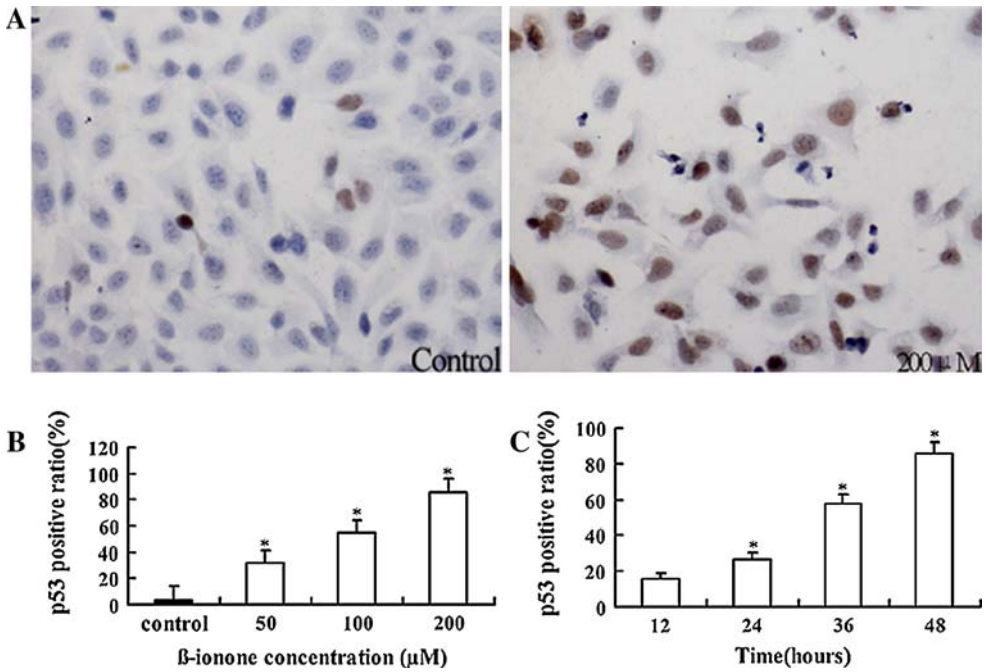

Fig. 6 Immunocytochemistry for p53. U2os cells were treated with 50, 100 or 200 μM *β*-ionone for 48 h and were stained with monoclonal antibodies against p53. The means and standard error of the mean were obtained from six independent experiments. Statistical analysis was performed by one-way ANOVA. **a** Immunocytochemical results for p53 in U2os cells. The *right* photograph shows that p53 was mainly expressed in the nucleus of U2os cells in response to 200 μM *β*-ionone (magnification: ×200). **b** The expression of p53 increased dose dependently in response to *β*-ionone treatment for 48 h ($P < 0.01$, $n = 6$). **c** The expression of p53 increased time dependently in response to 200 μM *β*-ionone

β-ionone-induced apoptosis in U2os cells, the p53, Bcl2 family (Bcl2 and Bax) and Caspase 3 were studied in our study. Our immunoblotting and immunocytochemical results of Bcl2 and Bax showed that Bcl2 was downregulated and Bax was upregulated after exposure to *β*-ionone for 48 h. The shift in balance between Bcl2 and Bax likely influenced cytochrome *c* release and apoptosis.

P53 is known to interact with Bax, by promoting its activation and insertion into the mitochondrial membrane. Cytosolic Bax is unable to induce cell death, and translocation of Bax to mitochondria is a critical step in p53-mediated apoptosis [18]. To further confirm the involvement of mitochondrial pathway in *β*-ionone-induced apoptosis, we confirmed the translocation of Bax and the release of cytochrome *c* from the mitochondria with the immunohistochemistry technique. Bax translocation may evoke leakage of mitochondrial cytochrome *c* into the cytoplasm, then promote the formation of an apoptosome complex by binding to apoptotic protease activating factor-1 (Apaf-1), which can recruit and activate Caspase 9. This then amplifies the downstream activation of the apoptotic cascade through the cleavage and activation of other effector caspases, such as Procaspase 3 and 7, which are responsible for the cleavage of various proteins leading to biochemical and morphological features characteristic of apoptosis [19–21].

The Caspase family of cysteine proteases plays a key role in apoptosis and Caspase 3 is an executioner caspase to induce apoptosis. Caspase 3 activation represents a critical and irreversible point in tumor development [22]. The activation process is carried out as follows: the upstream proteases (e.g. Caspases 8, 9 and 10) can synthesize Caspase 3 as an inactive proenzyme during the course of apoptosis, and the active forms of Caspase 3 are 17 and 21 kDa. The active Caspase 3 cleaves the key protein such as poly (ADP-ribose) polymerase (PAPP) to execute the whole apoptotic process [23, 24]. In our study, Caspase 3 expression at different times indicated that incubation of U2os with *β*-ionone for 48 h induced the greatest apoptosis in the U2os cells. The Caspase 3 activity assay and the expression of active Caspase 3 (17 kDa) confirmed that *β*-ionone induced cell apoptosis via Caspase 3 activation.

In our study, *β*-ionone activated p53, which upregulated Bax and downregulated Bcl2 to cause disruption of the mitochondrial membrane, which promoted Bax translocation and cytochrome *c* release from mitochondria into the cytosol, at a deeper level, activated the effector Caspase 3, to ultimately induce apoptosis. These observations confirmed that apoptosis induced by *β*-ionone was mediated by p53-dependent mitochondrial pathway.

However, *β*-ionone has been shown to affect the MAPK pathway in MDA-MB 435 cells [14]. The mechanisms

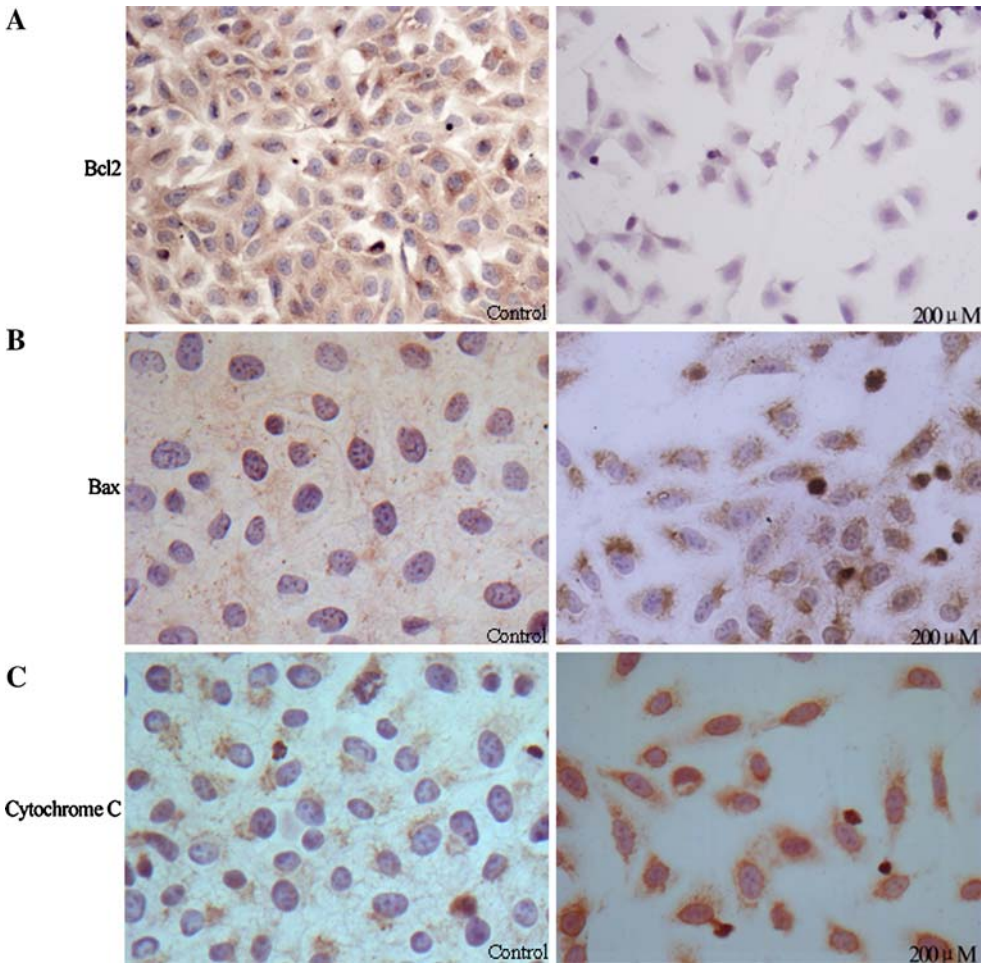

Fig. 7 Immunocytochemistry for Bcl2, Bax and cytochrome *c*. Cells treated with ethanol control or 200 μM *β*-ionone for 48 h were stained with antibodies against Bcl2, Bax or cytochrome *c*. **a** Immunocytochemical staining for Bcl2 showed that *β*-ionone decreased the expression of Bcl2 (magnification: **a** ×200; **b** ×400). **b** Immunocytochemical staining for Bax showed that *β*-ionone increased the expression of Bax (magnification: ×400). **c** The *right* figure shows that the expression of cytochrome *c* increased in response to *β*-ionone treatment for 48 h. U2os cells were treated with *β*-ionone for 48 h and cytochrome *c* was relatively diffusely expressed in cytosol whereas in cells treated with ethanol, cytochrome *c* was relatively concentrated in mitochondria (magnification: ×400)

involved in apoptosis in U2os and MDA-MB 435 cells were probably different because of respective tumorous specificity.

In conclusion, *β*-ionone can inhibit cell growth and proliferation and induce apoptosis of U2os cells. Our results indicate that *β*-ionone induces apoptosis in U2os cells via a p53-dependent mitochondrial signaling pathway.

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